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RESEARCH PAPER

Ovarian Histology and Histopathology of Olive Barb, *Puntius sarana* Exposed to Endocrine Disrupting Chemical (17-A Methyl Testosterone) in Laboratory Condition

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Abstract

17-α methyl testosterone is a major endocrine disrupting chemical (EDCs), reported harmful effect on endocrine system of aquatic animals. To study the impact of 17-αMT on olive barb *(Puntius sarana)* gonad, sixty fishes were collected for in vitro experiment. Hormonal treatments were carried out 90 days with three distinct hormonal dosages (T_1:40 mg/kg; T_2:60 mg/kg and T_3:90 mg/kg) mixed with pelleted feed and no hormone in control tank (C). Each 30 days intervals, histological analysis was performed to observe alterations in the gonad due to the effect of 17α-MT. Four gamete developmental stages of female *P. sarana* ovary were identified through microscopic examination named immature, previtellogenic, vitellogenic and mature stage. Mild to moderate histological abnormalities has been observed in ovary for different treatments. T1 showed apparently normal histology except some alterations like empty follicle (ef), lipid droplet (ld), vitelline envelop breakdown (veb), but pronounced alterations were detected at higher concentration T2 and T3, increasing with longer exposures for instance degenerated nucleus (dn), atretic follicle (af), thickened vitelline envelop (tve), residual oocyte (roc). This preliminary study revealed more profound responses to EDCs exposure in the olive barbs which could serve as a model bio-indicator in ecotoxicological study.

Introduction

Fish particularly holds significant importance for human health because of providing essential nutrients for our body and become an inimitable consuming item in the diet of our country's people. Among freshwater species, a well-known species *Puntius sarana* (Hamilton, 1822) known as “Olive barb”, is classified as highly endangered due to its ecological importance (IUCN Bangladesh, 1998; Ameen *et al.,* 2000) and become vulnerable in India (Mijkherjee *et al.,* 2002). The nutritional composition of olive barbs, (*P. sarana*) is impressive which contains double the amount of iron compared to many cultured carp species (Villif and Jorgensen, 1993).

The gonad of fish is the organ responsible to develop gamete and reproduction. Developing ovary is a long process and it is important for all species (Brown *et al.,* 2011). Successful reproduction mainly emphasised by female oocyte production more than a male sperm production (Trippel 2003; Helfman *et al.,* 2009). The reproduction process can be disturbed by several factors like chemicals. Among the chemicals, endocrine disrupting chemicals (EDCs) are one of the main agents to alter the normal physiological and hormonal regulations. Endocrine-disrupting compounds

(EDCs) are synthetic estrogens (17α methyl testosterone, 17β-estradiol, Ethinyl estradiol etc) which possibly divert the attention away from the delicate hormone pathways that control reproductive activities which has been imported in the waterbody from the neighbouring tannery effluents and other pharmaceutical industries. Available (EDC's) are responsible for disrupting the reproductive endocrine system which affects gamete development by altering the hormonal environment during gamete development. It also affects the sexual variation of the gonad, late sexual maturation, gonadosomatic index (GSI), reproductive tract and disrupts gonad components (Milnes *et al.,* 2006). There is no question that EDCs can have long-term consequences on wild fish populations' reproductive and future population development (Jobling, 2002b; Kidd, 2007; Karim *et al.,* 2022). Using these kinds of anabolic hormonal agents banned for causing harm on human, aquatic animals and surrounding environment (Suseno *et al.,* 2020). The effects of MT, the primary focus has been on the reproductive physiology of fish. Adult exposure has been shown to lesser Gonado-somatic Index (GSI), amend ovarian structure, disruption in follicle cell including steroid abnormality, decrease the fertility rate, also changes genetics engaged into sexual diversification (Van den Belt *et al.,* 2002; Segner *et al.,* 2003; Versonnen and Janssen 2004; Urbatzka *et al.,* 2012). Although huge scientific works have been conducted throughout the world to understand the effect of EDCs in aquatic animals as well as in the human body, yet Bangladesh is far away from this type of research. Bangladesh is new to this kind of work, even no work is done yet on the endocrine-disrupting chemicals. In this experiment we aimed to assess the effect of 17α methyl testosterone hormone on gonad of olive barbs at different dosages. We have selected Olive barb *(P. sarana)* as our experimental fish as it is available, economically important and has potential for ecotoxicological research.

Materials and Methods

Olive barb (*P. sarana*) Husbandry

The duration of the research was 90 days (3 months) (October/2020 to December/2020). Total 60 fishes, comprising an (mean weight 9.78±1.05 g and length of 8.64±0.83 cm) were randomly distributed to 4 prepared glass aquariums accommodating 15 fishes per aquarium (Table. 1). Prepared feed fed within 5% body weight of fishes. Therefore, fishes were transferred to glass aquarium (0.762 m× 0.371 m× 0.371 m) and a capacity of 60L water. All the aquariums were facilitated with an aerator for the purpose filtration of debris and thus proper supply of oxygen.

Feed Preparation

17α MT hormones (Sigma, St. Louis, MO) were used as EDCs. Commercial feed comprising 35% basic protein, 12.0% lipids, 8.5% ash, 7.6% fibres and wetness 9.36% was used for the feed preparation. The composition of feed was pelleted form. The manufacturing of MT-mixed-feed was done as described in (Teichert-Coddtngton et al. 2000). Diverse amounts of 17α MT (40 mg/kg for treatment 1, 60 mg/kg for treatment 2 and 90 mg/kg for treatment 3) were liquefied in 95% ethanol at a rate of 35 ml, 51 ml and 76 ml and the mixed solution was kept the whole night for perfect mixing and was kept at room temperature (25^0 C) (Table 1). The next morning the solution was sprayed over the pellet feed through a hand sprayer and mixed vigorously. Alcohol was evaporated from the feed as feeds were kept at room temperature. Feeding regime was maintained 2 times a day.

Estimation of Water Quality Parameters

Water quality parameters were recorded by the Multimeter (YSI Professional plus multiparameter water quality meter, USA) for 3 months (from October/2020 to December/2020). Each record was made daily at 9:00 am in the morning.

Histological Procedure

Each group's fish were taken out and sacrificed. The gonad was cut into small pieces and immediately fixed in a 10% Neutral buffer Formalin (40% Formaldehyde) solution. All fixed gonads were routinely processed using the paraffin embedding procedure after 2/3 days. Embedded tissues were sectioned at 5-7µ in thickness and then stained with Haematoxylin and Eosin dye (H & E). Prepared mounted slides of the gonad of Olive barb were studied under an electronic microscope using 10**X**, 40**X** with a suitable magnification. Photographs of the histological slides were taken using a connected digital camera with the microscope (Optika Microscope camera; model B9, Italy).

Table 1: Calculation of experimental fish feeds.

No. of Fishes	Treatment	Feed % body weight	Alcohol (95% Ethanol)	17α MT (mg/kg feed)
15	T_1	5	35 ml	40
15	T_2	5	51 ml	60
15	T_3	5	76 ml	90
15	C	5	-	0

T1: treatment 1; T2: treatment 2; T3: treatment 3 and C: Control

Statistical Analysis

All the data were coded and recorded in a Microsoft Excel sheet. Data were presented as mean ± SD. Statistical analyses were performed using the SPSS version 21.0, Inc. Chicago, IL, USA. Data were tested for normality using the Kolmogorov-Smirnov test. One-way ANOVA was applied for the normally distributed data. If the variances were not normally distributed, Mann-Whitney U post hoc test was applied. Significance level was set to P≤0.05.

Result

Water Quality Parameters

During the in vitro experiment, water quality parameters were recorded for 3 months (from October/2020 to December/2020) at the monthly interval. All the water quality parameters were in a suitable range for fish. No significant differences were found among the investigated parameters during the study period (Table 2).

Histopathological Examination

Developmental Stages of Gamete

Four different stages of gamete development were identified in the female fishes named i) immature stage, ii) vitellogenic stage-1, iii) vitellogenic stage-2 and iv) maturation stage. Immature ovary contains a lot of peri nuclear oocytes (Poc) early preparatory phase with nucleus (N), also called Previtellogenic stage, while vitellogenic stage-1 contained initial primary oocyte and primary Oocyte with early vitellogenesis. Vitellogenic stage-2 were developing large nucleus (Ln) and a lot of developing cortical alveoli (CA) yellow dashes and yolk granules (Yg) in cytoplasm and mature oocyte were with ovarian lumen (Ol) and a little peri-nuclear oocytes (PeO) (Figure 1).

Table 2: Water quality parameters during the investigation

Parameters	Time Period (days)	C	T_1	T_2	T_3
Temperature (°C)	90	21.6±1.25^a	22.5±1.27^a	22.3±1.75^a	22±1.8^a
DO (mg/l)	90	6.87±0.85^a	6.37±0.7^a	6.67±1.0^a	6.59±1.25^a
p^H	90	7.06±0.5^b	7.35±0.03^a	7.80±0.02^a	7.68±0.25^a
TDS (mg/l)	90	93.5±2.5^a	94.6±2.77^a	95.8±2.71^a	93.5±3.54^a
Ammonia (NH_3)	90	0.5±1.0^b	1.5±1.0^a	1.5±1.0^a	2.0±1.0^a
Salinity (PPT)	90	0.7±0.5^a	0.773±0.01^a	0.86±0.25^a	0.963±0.1^a
Conductivity (us/cm)	90	154.3±4.29^b	172.7±2.13^a	235.7±5.20^a	284.4±4.5^a

Data are presented as mean ± SD. Different letters indicate significance difference among investigated groups (P<0.05).

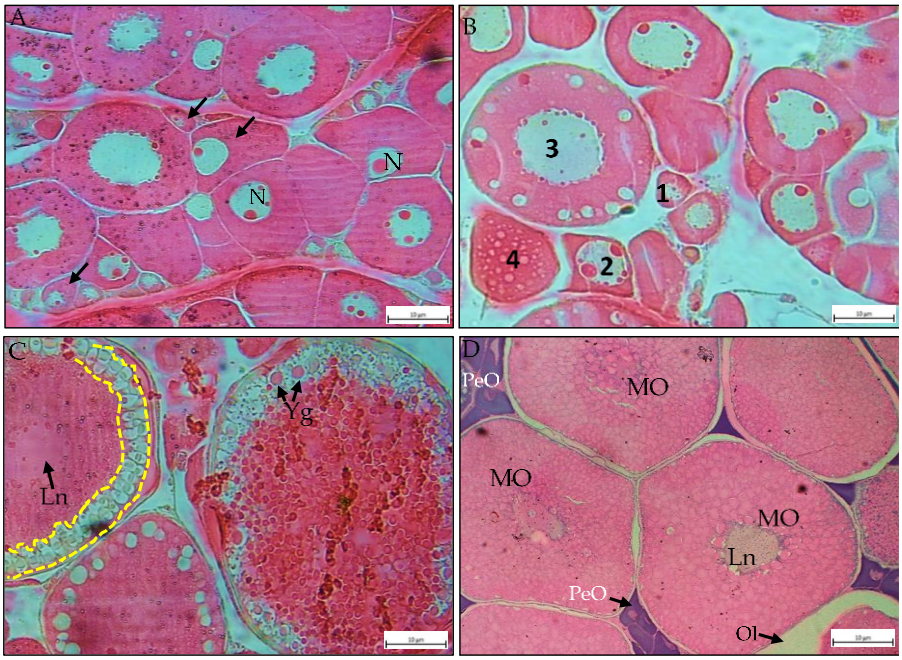

Figure 1. Development stages of female P. sarana ovary (H/E stain). A. Immature ovary contains a lot of peri nuclear oocytes (Poc) (→) early preparatory phase with nucleus (N) each, (Previtellogenic stage). B. Development continuing with (1) Initial Primary Oocyte; (2) Primary Oocyte (3) Pre Vitellogenic-Oocyte; and (4) Early Vitellogenic-Oocyte are present, (Vitellogenic-1 stage). C. Developing large nucleus (Ln) and a lot of developing cortical alveoli (CA) yellow dashes and yolk granules (Yg) in cytoplasm, (Vitellogenic-2 stage). D. Ovarian lumen (Ol) with Mature oocytes (MO) and a little Peri-nuclear oocytes (PeO), (Maturation phase) (Scale = 10 µm, H&E).

Histological Changes in the Ovary of *P. sarana* Fed with Experimental Diets

After the treatment of 17 alpha methyltestosterone for 90 days, olive barb *(P. sarana)* was collected from (Treatment 1, 2 and 3) containing three different dosages of the hormone for assessing the effect of hormone for gonadal maturation and observed under a light microscope to compare the possible changes that took place in the gonad and histopathological changes were marked in the gonad images.

Histological alterations and growth parameters in the treated groups were summarized in (Table 3 and 4). Control fishes used in this experiment showed no alterations in the histological alterations during the histological observations and all fishes were remain unchanged during the whole experiment. At the same time, three different treatment trails showed various alterations during the observations. After the observations of 90 days trial when the histological slides were observed under the microscope, it shows that Treatment 1 (T_1) with 40 mg/kg (MT) contains mild (<10%) changes marked as (+) with a variety of

Table 3: Histopathological alterations observed in ovary throughout the study period in different treatment trials.

Anomalies	Treatments	Days		
	17α-MT (mg/kg)	30	60	90
Nucleus (N)	C	-	-	-
	T1	+	+	+
	T2	+	++	++
	T3	+	++	+++
Atretic follicle (Af)	C	-	-	-
	T1	-	+	+
	T2	+	+	++
	T3	+	++	+++
Cortical alveoli (Ca)	C	-	-	-
	T1	+	+	++
	T2	+	++	+++
	T3	+	++	+++
Empty follicle (Ef)	C	-	-	-
	T1	-	+	+
	T2	+	+	++
	T3	+	++	+++
Lipid droplet (Ld)	C	-	-	-
	T1	-	+	+
	T2	-	+	++
	T3	-	+	+++
Vitelline envelop breakdown (VeB)	C	-	-	-
	T1	-	+	+
	T2	-	+	+
	T3	-	++	+++
Mature oocyte (Mo)	C	-	-	-
	T1	-	-	+
	T2	-	+	++
	T3	+	+	+++
Mature follicle (Mf)	C	-	-	-
	T1	-	+	+
	T2	-	+	++
	T3	+	+	+++
Thickened vitelline envelop (TVe)	C	-	-	-
	T1	-	-	-
	T2	-	+	+
	T3	+	+	+++
Secondary oocyte (So)	C	-	-	-
	T1	-	-	+
	T2	-	+	++
	T3	+	++	+++
Yolk globuline (Yg)	C	-	-	-
	T1	-	+	++
	T2	+	+	+++
	T3	+	++	+

C: Control; T1: treatment 1; T2: treatment 2 and T3: treatment 3. -: Normal (0%); +: mild (< 10%); ++: moderate (10 to 50%) and +++: Severe (> 50%) (adapted from Hossain et al., 2022).

alterations. Regarding all the results, it makes a clear comparison between the three treatment trials, where treatment 1 was less diversified with histological alterations and the maximum histological alterations were identified in the treatment 3 trial as it was very severe with complications.

Discussion

Exact evaluation of ovary and maturation time and oocyte development steps are very sensitive to be described yet (Alonso-Fernandez, 2011). This experiment reported four different gamete development stages during oogenesis like previtellogenic stage; (Figure 1a); vitellogenic-1 stage (Figure 1b); vitellogenic-2 stage (Figure 1c); Maturation phase (Figure 1d), all stages were in agreement with previous researches (Wallace Selman, 1981; West, 1990; Lucano-Ramirez *et al.,* 2001; Saborido and Kjesbu, 2012; Sultana *et al.,* 2022). Histological changes in the ovary of an adult olive barb observed after feeding 17α-MT hormone mixed feed, a similar study done by Ahmad *et al.,* 2002 reported the harmful effect of 17α MT on the gonads of Nile tilapia *(Oreochromis niloticus)* which is regarded as a model fish for research. Our 90-day experiment, the development of peri nucleolar oocytes (PoC) (Figures 2a, 3a, 4a) got visible which was in the previtellogenic stage and by the course of time nucleolar (N) (Figure 3a, c); lipid droplet (Ld) (Figure 2d; 3b, 4c) stage becomes developed and turned into the vitellogenic stage (Vt), supports the findings with where vitellogenesis stage is considered with a very important lengthy stage happens after previtellogenesis (Brown-Peterson *et al.,* 2011). In addition, cortical alveoli (Ca)

Table 4: Effect of orally-administrated 17α-Methyl Testosterone on growth parameters and survival rate of *P. sarana* after 90 days of treatment.

Treatment	Growth parameters		Survival rate (%)
	Length (cm)	Weight (g)	
C	8.5 ± 0.66^{b}	8.37 ± 1.18^{ab}	100
T1	8.71 ± 1.4^{ab}	7.68 ± 0.43^{b}	87
T2	9.1 ± 0.65^{a}	8.6 ± 0.39^{ab}	76
T3	9.2 ± 0.35^{a}	9.66 ± 0.62^{a}	68

Data are presented as mean ± SD. Different letters indicate significance difference among investigated groups (P<0.05). C: control; T1: treatment 1; T2: treatment 2; T3: treatment 3.

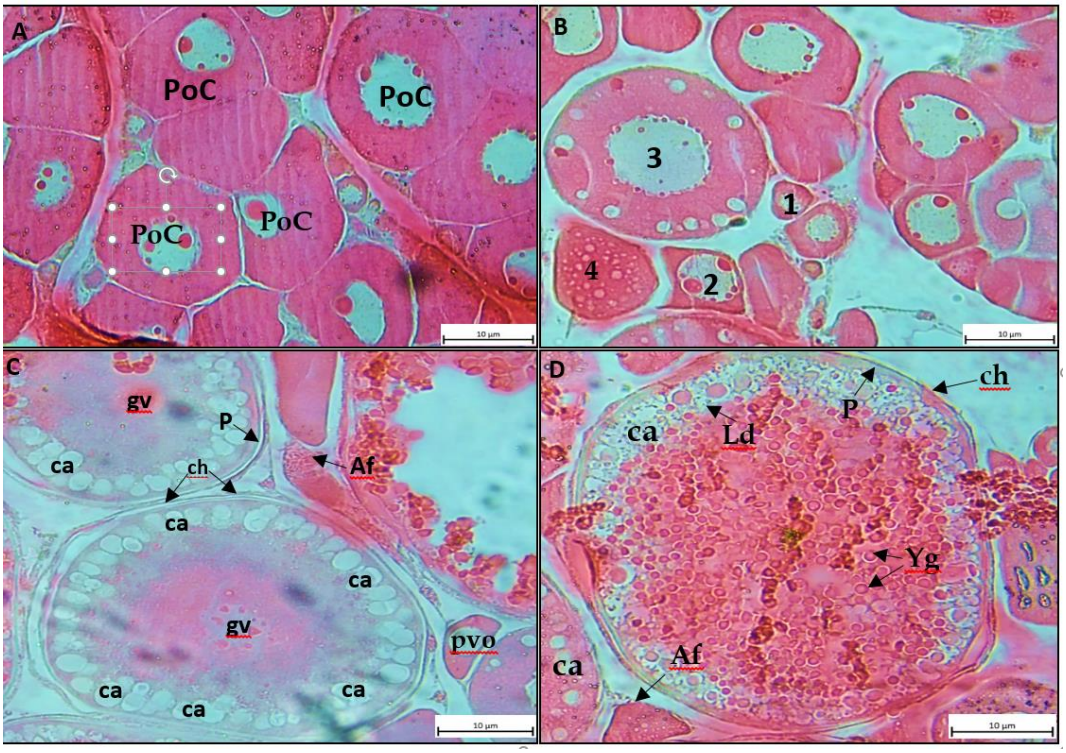

Figure 2. Photo micrograph of 30 days after 17α MT Hormone exposure- A. Control fish ovary with organized peri nucleolar oocytes (PoC). B. T1 (40 mg/kg 17α MT) showed different stages of ovarian development, (1) Initial Primary Oocyte; (2) Primary Oocyte later stage, the onset of cortical alveoli formation; (3) Pre Vitellogenic-Oocyte; and (4) Early Vitellogenic-Oocyte are present. C. T2 (60 mg/kg 17α MT) contains well-organized cortical alveoli (Ca), Atretic follicle (Af), numerous yolk granule (Yg); Chorion (Ch); Previtelin (P), previtellogenic (Pvo), Germinal vesicle (Gv); D. T3 (90 mg/kg 17α MT) contains whitish Lipid droplet (Ld). (Scale = 10 µm, H&E).

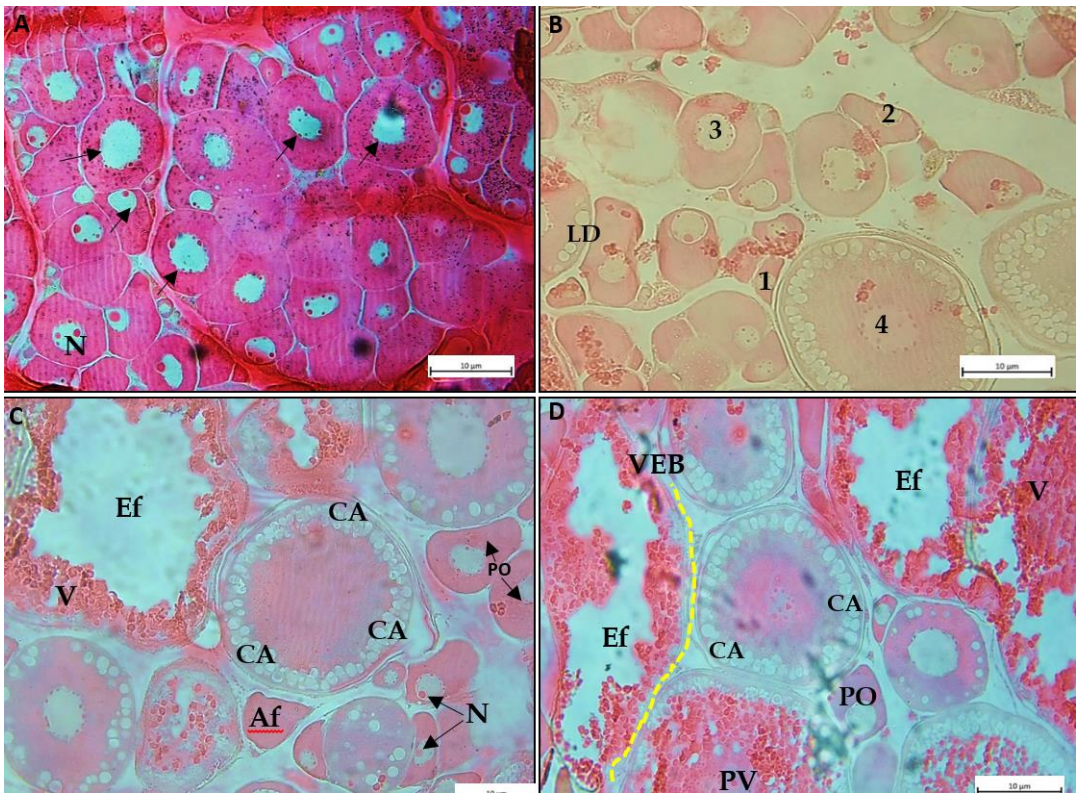

Figure 3. Ovary of P. sarana observed after 60 days of 17α MT Hormone exposure. A. Control trial Oocytes are highly developed and well ornamented with peri nucleolar oocytes (PoC) (→). B. T1: Lipid droplets (Ld), enlarged cortical alveoli (Ca), Oocytes shrinked, nucleus disappeared. C. T2: Nucleoli (N) (→), Vitellogenic (V) and Empty follicle (Ef). D. T3: Increased cortical alveoli (CA) are more visible and Vitelline envelop break down (VEB) yellow curved line (dotted pattern), negligible quantity of Peri nucleolar oocyte (PoC), empty follicle (Ef) (Scale = 10 µm, H&E).

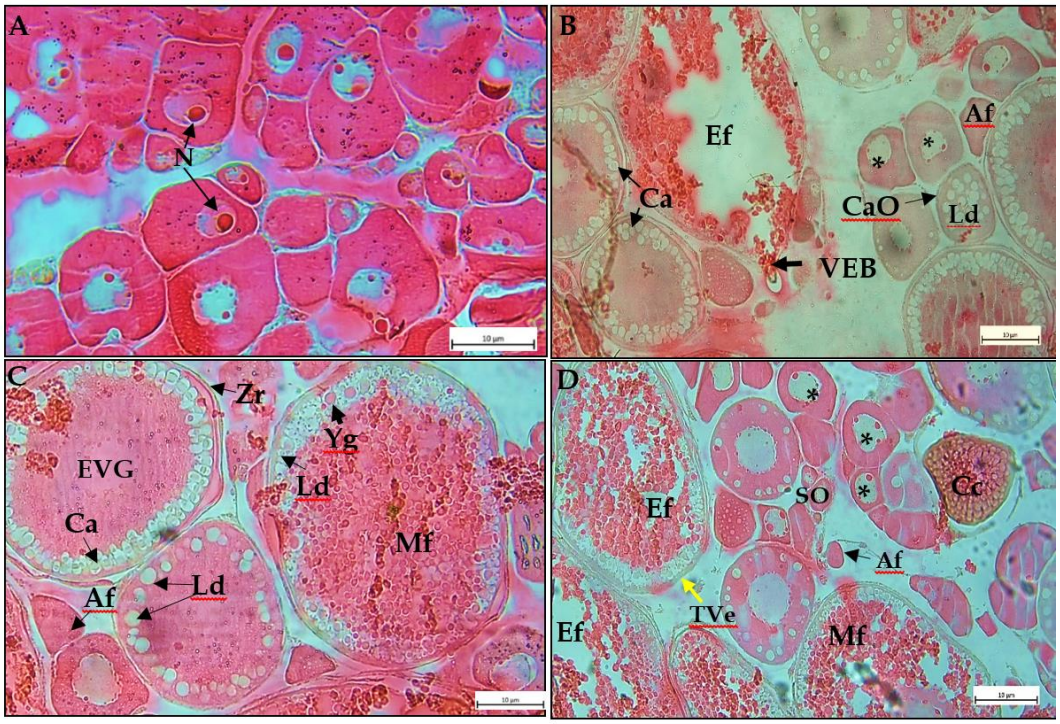

Figure 4. Photomicrograph of ovary after 90 days of 17α MT Hormone exposure. A. Control: Prei nucleolar oocytes (POc). B. T1: Cortical Alveolar Oocyte (CaO) with Vitelline Envelope breakdown (VEB) and Atratic follicle (Af) with a large area of empty follicle (Ef), Corticol Alveoli (Ca). C. T2: Mature follicular (Mf) stage with enlarged vitellogenic (EVG) visible with several Lipid droplets (Ld), Zona radiata (Zr), Yolk globuline (Yg). D. T3: Secondary Oocyte (*), empty follicle space (Ef) observed, Mature follicle (Mf), Thickened Vitelline envelope (TVe) and Cytoplasmic clumping (Cc) (Scale = 10 µm, H&E).

were seen as dominant numbers in 60 mg/kg feed eventually cortical alveoli are technically not the yolk since they do not nourish the embryo. Ahmad *et al.,* 2002 observed the effect of MT at a higher dosage (5, 10, 20 and 40 mg MT/kg feed) over GSI (Gonado-somatic Index) resulted in a dramatic reduction in both male and female fish meanwhile, minute changes were observed at a little number of MT dosages where it maintained (0.5, 1.0 and 2.5 mg MT/kg feed). So, it meant that GSI varied with the dosage of t he MT hormone. Shen *et al.,* (2015) examined 17α Methyl testosterone for its effects on sex reversal, gonadal anatomy, and development in yellow catfish *(Pelteobagrus fulvidraco),* where high dose was attributed to testicular degeneration. A similar experiment was conducted by Van Den Belt *et al.,* 2002, who used Zebrafish *(Danio rerio)* to nourish with 17α-ethynyl estradiol (a synthetic analogue of natural estrogen) and oral contraceptives containing 17β estradiol enhanced the number of atretic follicles (Af) (Figure 2c; 4b) and increased number of mature ooctytes (MO) (Figure 1d) and degenerations like empty follicle (Ef) (Figure 3c,d; 4b,d); vitelline envelop breakdown (VEB) (Figure 3d, 4b); in comparison to control fish, similar with our findings as we observed several atretic follicles in different dosage, excessive dosage of MT makes very unpleasant changes in the ovarian structure of *P.sarana*. A good number of analytical works on the morphological assessment on gonads, analysis of fertility-fecundity and also on the changing behaviour difference caused by the exposure of estrogenic compounds (Brion *et al.,* 2004; Maack and Segner, 2003; Nash *et al.,* 2004; Schäfers *et al.,* 2007; Van den Belt *et al.,* 2003). All these relevant frameworks and the observation of histop athological alterations into the ovary through the exposure of EDC undoubtedly showed hazards in fish populations and imbalanced the regular ecosystems. However nowadays, histological justification has become a great criterion for identifying the oocytes stages of specific-species for comparative purposes (Lowerre-Barbieri *et al.,* 2011a). For instance, Komen *et al.,* (1989) reported that 17α-MT delivered a various dosage of 50 and 100 mg/L to *Cyprinus carpio* resulted to a severe malformation in fish gonad and higher dosage caused sterility of fish reproduction. Sacobie and Benfey (2005) found similar annotations on *Oreochromis mossambicus* using 17α-MT at a dosage of 50 mg/L and the histology pattern resulted a lot of germ cells degenerations and gonad deformities. So, all endocrine disturbances in the waterbody, as well as the ecosystems, have clarified that open-water fish are at great risk due to EDCs exposures.

Conclusion

Endocrine disruptors like 17α-MT hormone are pervasive compounds and have been detected in aquatic environments across the world. Every scientific article has made its suggestion on EDC's and homologous products showed a similar mode of action which severely affected fish reproductive physiological. 17α-MT has been reported as a harmful effect on neuroendocrine system also it has been found an adverse effect on disorders of the reproduction system such as irregular spawning time, changes in reproductive physiology. To resolve this concern, there is an urgent need to assess the ecological status of water bodies to use an integrative ecosystem approach that links water contamination with its effects on aquatic life.

Ethical Statement

All experimental animal protocols in this study were reviewed and approved by the Ethical Review Committee of Sylhet Agricultural University, Sylhet, Bangladesh. The fish were handled with care and compassion at all the stages.

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